

APH-1a Is the Principal Mammalian APH-1 Isoform Present in γ -Secretase Complexes during Embryonic Development

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APH-1 (anterior pharynx defective) along with nicastrin and PEN-2 (presenilin enhancer) are essential components of the presenilin (PS)-dependent γ -secretase complex. There exist three murine *Aph-1* alleles termed *Aph-1a*, *Aph-1b*, and *Aph-1c* that encode four distinct APH-1 isoforms: APH-1aL and APH-1aS derived from differential splicing of *Aph-1a*, APH-1b, and APH-1c. To determine the contributions of mammalian APH-1 homologs in formation of functional γ -secretase complexes, we generated *Aph-1a*^{-/-} mice and derived immortalized fibroblasts from these embryos. Compared with littermate controls, the development of *Aph-1a*^{-/-} embryos was dramatically retarded by embryonic day 9.5 and exhibited patterning defects that resemble, but are not identical to, those of *Notch1*, *nicastrin*, or *PS* null embryos. Moreover, in immortalized *Aph-1a*^{-/-} fibroblasts, the levels of nicastrin, PS fragments, and PEN-2 were dramatically decreased. Consequently, deletion of *Aph-1a* resulted in significant reduction in levels of high-molecular-weight γ -secretase complex and secretion of β -amyloid (A β). Importantly, complementation analysis revealed that all mammalian APH-1 isoforms were capable of restoring the levels of nicastrin, PS, and PEN-2, as well as A β secretion in *Aph-1a*^{-/-} cells. Together, our findings establish that APH-1a is the major mammalian APH-1 homolog present in PS-dependent γ -secretase complexes during embryogenesis and support the view that mammalian APH-1 isoforms define a set of distinct functional γ -secretase complexes.

Key words: *Aph-1a* null mice; APH-1 isoforms; γ -secretase complexes; nicastrin; presenilins; Alzheimer's disease

Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized by the deposition of β -amyloid (A β) and the presence of neurofibrillary tangles in the brain (Price and Sisodia, 1998; Petersen, 2003). A β peptides are generated by sequential endoproteolytic cleavages of β -amyloid precursor protein (APP) by BACE1 (β -site APP-cleaving enzyme) and γ -secretase complex (Wong et al., 2002). The presenilins (PSs), which when mutated cause autosomal dominant AD (Sisodia and St. George-Hyslop, 2002), are critical for the regulated intramembrane proteolysis of a growing number of type I transmembrane proteins, including APP and Notch (Selkoe and Kopan, 2003; Wolfe and Kopan, 2004). Recent studies (Edbauer et al., 2003; Farmery et al., 2003; Kim et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003) support the view that PSs form high-molecular-weight complexes with several other transmembrane proteins critical for γ -secretase activity, including nicastrin (NCT) (Yu et al., 2000; Li et al., 2003), APH-1 (anterior pharynx defective) (Francis et al., 2002; Goutte et al., 2002; Lee et al., 2004), and PEN-2 (presenilin enhancer) (Francis et al., 2002).

One essential member of γ -secretase complex is APH-1, a seven-pass membrane protein (Francis et al., 2002; Fortna et al., 2004) that

forms a stable subcomplex with nicastrin (Gu et al., 2003; Hu and Fortini, 2003; LaVoie et al., 2003; Takasugi et al., 2003). Unlike *Caenorhabditis elegans* and *Drosophila* in which a single *Aph-1* gene exists, three *Aph-1* genes are found in mice; *Aph-1a* is localized to chromosome 3, whereas *Aph-1b* and *Aph-1c* are arranged in tandem (~20 kb apart) on chromosome 9. Because two isoforms of APH-1a can be generated from alternative splicing of *Aph-1a*, APH-1a long form (APH-1aL) and APH-1a short form (APH-1aS), a total of four distinct APH-1 isoforms can be found in mice. Although APH-1a has ~55% similarity with APH-1b/APH-1c, whereas APH-1b and APH-1c share 95% similarity, it remains undefined whether different mammalian APH-1 isoforms possess similar or distinct roles in γ -secretase activity or as to their relative contributions to γ -secretase complexes during development, maturation, and aging.

To begin to assess the physiological roles of mammalian APH-1 isoforms, their contributions to formation of functional γ -secretase complexes, and the mechanism whereby APH-1 facilitates the complex assembly, we generated and analyzed *Aph-1a*-deficient mice and immortalized fibroblasts derived from *Aph-1a*^{-/-} embryos. *Aph-1a*^{-/-} embryos display phenotypes that resemble, but are not identical to, those of *Notch1* (Swiatek et al., 1994; Conlon et al., 1995; Huppert et al., 2000), *nicastrin* (Li et al., 2003), or *PS* (Donoviel et al., 1999; Herreman et al., 1999) null embryos. Importantly, the deletion of *Aph-1a* led to a significant reduction in nicastrin, PS, and PEN-2, accompanied by decrease in high-molecular-weight γ -secretase complex and secretion of A β peptides. Moreover, complementation analysis using *Aph-1a*^{-/-} cells showed that all murine APH-1 isoforms are functional in γ -secretase activity. Thus, our results establish that APH-1a is the principal APH-1 isoform present in γ -secretase

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complexes during mouse embryogenesis and support the view that mammalian APH-1 isoforms define a set of distinct functional γ -secretase complexes.

Materials and Methods

Cloning and transfection. *Aph-1aS* and *Aph-1b* cDNAs were generated by reverse transcriptase-PCR of mRNA extracted from tail of mice using the following primers: 5'-CAGTCCCTTTTCGCCTTCTTG, 5'-GTCC-TTACACGAAAGGCTGCGC, 5'-ATGACGCTGCCTGTGTCTTCG, and 5'-TCTGGAGCGT TGGTTGTAAGAAGAAAATC. *Aph-1aL* EST (expressed sequence tagged) clone was purchased from Invitrogen [EST library (San Diego, CA)]. All *Aph-1* cDNAs were then subcloned into pCDNA3.1-V5/His vector using a Topo expression kit (Invitrogen). *Aph-1a*^{-/-} cells were transfected with expression vectors encoding APH-1aL, APH-1aS-V5, APH-1b-V5, or APH-1c using the Lipfectamine 2000 reagent (Invitrogen). Cells were collected 48 hr after transfection and analyzed by protein blotting.

Generation of *Aph-1a*^{-/-} mice. The *Aph-1a* gene, isolated from a murine genomic 129/SvJ library (Stratagene, La Jolla, CA) using a murine *Aph-1a* cDNA probe (reverse transcriptase-PCR extracted from tail of mice using the primers 5'-GTTATCATCTG GTGGCGGAG and 5'-GCTGAAGTCAGGAAGTAATA GGGTGAAGTC) was characterized by a series of restriction enzyme and DNA sequencing analysis. *Aph-1a* genomic sequence was obtained from the National Center for Biotechnology Information mouse genome database. In the *Aph-1a* targeting vector, a 2.5 kb fragment containing exon 1 to intron 5 and part of exon 6 of the *Aph-1a* gene was replaced with a neomycin-resistant (*Neo*) gene. The linearized *Aph-1a* targeting vector was electroporated into 129/SvJ embryonic stem (ES) cells, and targeted clones were screened by Southern blot analysis using a flanking probe generated by PCR of mouse DNA via primers 5'-GAATTCAGTCTTCTCCCGC and 5'-CCTCTCGAAGTGTACCCCG.

Eight independent targeted clones were injected into C57BL/6 mouse blastocysts to generate *Aph-1a* chimeric mice. Mating of chimeric mice to C57BL/6 mice produced offspring bearing one inactivated *Aph-1a* allele (*Aph-1a*^{+/-} mice), and intercrosses of *Aph-1a*^{+/-} mice generated *Aph-1a*^{-/-} mice. DNA extracted from tail clips or yolk sacs of mice were genotyped by PCR using the following sets of primers: for the endogenous allele, 5'-GCTGTCTCTGCTTCTACAGGAG and 5'-CGGAA-GATCACCCATCTCCATCC; and for the targeted allele, 5'-GTACAC-CATCACCCGACTGTC and 5'-CTACCCGCTTCCATTGCTCAG.

Characterization of the *Aph-1a*^{-/-} embryos. Embryos were dissected and examined by light microscopy or fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), or processed for whole-mount *in situ* hybridization using *Dll1* (Delta-like 1) as probe generated using a Digoxigenin RNA labeling kit (Roche, Indianapolis, IN) following previously established protocols.

Generation of immortalized *Aph-1a*^{-/-} fibroblasts. Primary fibroblasts were cultured from *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} embryos at embryonic day 9 (E9) to E9.5. Briefly, the embryos were minced, suspended in 0.25% trypsin, and incubated at 37°C for 15 min. The tissues were then transferred to DMEM supplemented with 10% fetal bovine serum and dissociated by repeated trituration. The dispersed cells were plated in one well of a 48-well plate and subsequently transfected with large T antigen (Cai et al., 2001). Immortalized cells were then passaged and frozen in liquid nitrogen for later use.

Antibodies and protein blotting. Protein extracts were resolved on 4–20% Tris-glycine or 10–20% tricine gels, transferred to polyvinylidene difluoride membranes, and probed with the following antibodies: anti-human APH-1aL and anti-human APH-1b/c (1:1000; Covance, Princeton, NJ); anti-nicastrin (NCT-3925; 1:2000) (Li et al., 2003); anti-PS1-C-terminal fragment (CTF) (1:2500), anti-PS1-N-terminal fragment (NTF) (1:2000), and anti-PS2-CTF (1:2000) (Thinakaran et al., 1996); anti-PEN-2 (1:2000) (Vetrivel et al., 2004); anti-APP-CTF (CT15, 1:2000) (Chemicon, Temecula, CA); anti-V-5-HRP (1:2000) (Invitrogen); rabbit anti-superoxide dismutase (SOD1) antibody (1:5000; previously generated in our laboratory); and mouse monoclonal antibody against actin (Chemicon). Protein extracts were analyzed by immunoblotting with enhanced chemiluminescence.

Blue native-PAGE analysis. To prepare the membrane fraction, cells were washed with PBS, resuspended in 20 mM HEPES, pH 7.2, with 150 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and a mixture of protease inhibitors (Roche), and homogenized with 30 strokes in a glass Dounce homogenizer. Cell debris and nuclei were removed by centrifugation at 800 × g for 10 min. The supernatants were centrifuged at 100,000 × g for 60 min to collect the membrane fractions. Membrane preparations were resuspended in 20 mM Tris HCl, pH 7.4, with 0.5% N-dodecyl β -D-maltoside, 500 mM e-amino caproic acid, 2 mM EDTA, and 10% glycerol, incubated on ice for 60 min, and then centrifuged at 100,000 × g for 60 min. The membrane protein extracts were subjected to blue native-PAGE essentially as described previously (Schagger and von Jagow, 1991). Marker proteins were BSA (66 kDa and dimer at 132 kDa), β -amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (667 kDa) (Sigma, St. Louis, MO). After electrophoresis, protein complexes were analyzed by immunoblotting with antisera specific to nicastrin and PS1.

AP assays. APP recombinant adenovirus expressing human APP Swedish mutation (APPSwe) was constructed as described previously (Cai et al., 2001). To examine APP processing, immortalized fibroblasts were infected with 5 × 10⁶ plaque-forming units of adenovirus for 24–36 hr. A β 40 and A β 42 levels from culture supernatants of cells were measured using a quantitative sandwich ELISA kit (Biosource International, Camarillo, CA) that specifically detects human A β 40 or A β 42.

Results

Aph-1a-deficient mice are embryonic lethal

To determine the physiological role of *Aph-1a* and to assess whether different mammalian isoforms of APH-1 confer functional differences in γ -secretase activity, we examined functional consequences of ablating *Aph-1a* in mice by using a homologous recombination strategy in ES cells to inactivate this gene.

To generate the *Aph-1a* targeting vector, a 2.5 kb fragment containing exon 1 to intron 5 and part of exon 6 was replaced with a neomycin-resistance gene (Fig. 1A). 129/SvJ ES cells were transfected with the linearized *Aph-1a* targeting vector, and 19 clones (of 220 screened) were targeted at the *Aph-1a* locus (Fig. 1B). Eight ES cell clones with a targeted *Aph-1a* allele (*Aph-1a*^{+/-}) were injected into C57BL/6 blastocysts to generate *Aph-1a* chimeric mice. Mating of chimeric mice to C57BL/6 mice produced *Aph-1a*^{+/-} mice. However, genotypic analysis of postnatal progeny from intercrosses of *Aph-1a*^{+/-} mice revealed the presence of only *Aph-1a*^{+/+} and *Aph-1a*^{+/-} pups [with an *Aph-1a*^{+/+} to *Aph-1a*^{+/-} mice ratio of 0.5 (Table 1)], suggesting that *Aph-1a* knock-out mice might be embryonic lethal.

To determine the age and stage at which embryos died, we collected embryos from E8 to E16. Whereas *Aph-1a*^{+/+} and *Aph-1a*^{+/-} embryos were identified during these time points (Fig. 1C), no *Aph-1a*^{-/-} embryos can be recovered after E11 (Table 1). RNA analysis from yolk sacs of *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} embryos confirmed the absence of *Aph-1a* mRNA from *Aph-1a*^{-/-} embryos (Fig. 1D).

APH-1a is the major mammalian APH-1 isoform required for Notch signaling during embryogenesis

To determine whether the *Aph-1a* null phenotype resembles that of *Notch1* (Swiatek et al., 1994; Conlon et al., 1995; Huppert et al., 2000), *nicastroin* (Li et al., 2003), or *PS* (Donoviel et al., 1999; Herreman et al., 1999) null embryos, we undertook a series of morphological studies to characterize the embryonic phenotypes of *Aph-1a*^{-/-} embryos. The development of *Aph-1a*^{-/-} embryos was dramatically retarded by E9.5 when compared with *Aph-1a*^{+/+} and *Aph-1a*^{+/-} littermate controls (Fig. 2A,B), and growth is arrested by E10.5 (Fig. 2C). Although some E11 *Aph-1a*^{-/-} embryos can be recovered, no heartbeats can be detected

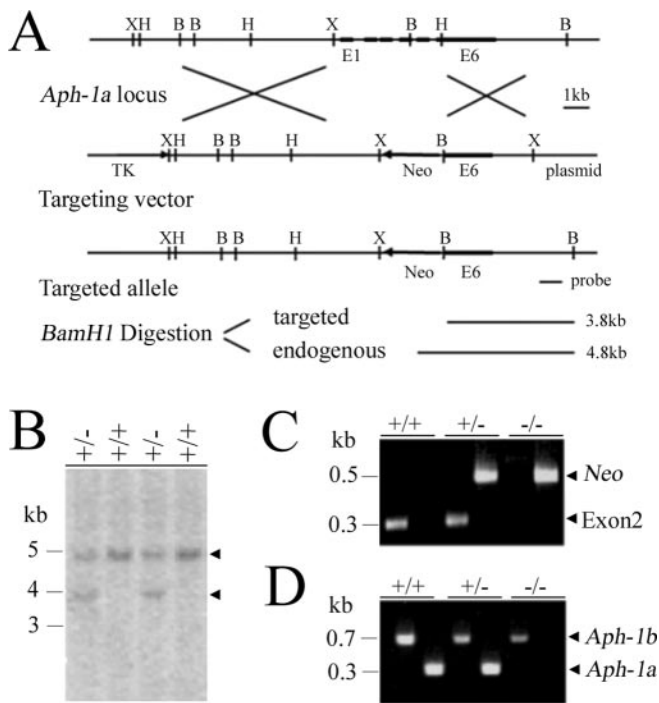


Figure 1. Targeted disruption of the *Aph-1a* gene by homologous recombination. *A*, Maps of the *Aph-1a* locus, the targeting vector, and the disrupted *Aph-1a* allele. Black boxes indicate exons 1 through 6. Neo, Neomycin gene; TK, HSV thymidine kinase gene; X, *Xba*I; H, *Hind*III; B, *Bam*HI. Lines below the maps denote expected sizes for *Bam*HI-digested fragments detected by a 3'-flanking probe (black bar) for targeted and endogenous *Aph-1a* alleles. *B*, Analysis of genomic DNA from ES cells by Southern blot. The *Bam*HI fragments detected for endogenous (top arrowhead, 4.8 kb) and targeted (bottom arrowhead, 3.8 kb) *Aph-1a* alleles with the 3' probe are indicated. *C*, PCR analysis of yolk sac DNA from embryos at E9.5 by PCR using specific sets of primers to detect endogenous (Exon2) and targeted (Neo) *Aph-1a* alleles. *D*, Total RNA extracted from E9.5 *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} embryos were subjected to reverse transcriptase-PCR analysis using primers specific for either *Aph-1a* or *Aph-1b* RNA.

Table 1. Progenies of crosses of *Aph-1a*^{+/-} mice

Age	Number of litters	Total number of pups	<i>Aph-1a</i> ^{+/+}	<i>Aph-1a</i> ^{+/-}	<i>Aph-1a</i> ^{-/-}
E8–E8.5	3	21	4	11	6
E8.5–E9	7	78	22	40	16
E9–E9.5	6	56	15	29	12
E9.5–E10.5	10	75	18	40	17
E10.5–E11	4	31	8	14	9
E11–E12	3	24	9	15	0
E12–E16	5	34	10	24	0
Adult	6	35	11	24	0

Number of embryos is shown for each genotype at various time points indicated. No *Aph-1a*^{-/-} embryos were recovered after E11.

from these embryos. Similar to *Notch1* null embryos, defects in vascular morphogenesis of the yolk sac (Fig. 2*A*), distention of the pericardial sac (Fig. 2*B,C*), underdeveloped first branchial arch and heart chambers (Fig. 2*D,E*), and kinks in the caudal part of the neural tube (Fig. 2*H*) were observed in *Aph-1a*^{-/-} embryos. However, in contrast to *Notch1*, *nicastrin*, or *PS* null embryos, defects in somite patterning seen in *Aph-1a*^{-/-} embryos (Fig. 2*F,G*) were less severe, particularly the rostral somites, consistent with the finding that *Aph-1a*^{-/-} embryos survive 1 d longer than *Notch1*, *nicastrin*, or *PS* null embryos. These results indicate that the presence of APH-1b and APH-1c isoforms accounts for the differences in the phenotype observed between *Notch1* and *Aph-1a* null embryos.

Because Dll1, a ligand of Notch1, is misregulated in the neural tube of *PS* null embryos (Donoviel et al., 1999), we predict that such an outcome would be observed in *Aph-1a*^{-/-} embryos. Whole-mount *in situ* hybridization analysis showed that the level of *Dll1* mRNA is indeed upregulated in neural tubes of E8.5 *Aph-1a*^{-/-} embryos (Fig. 2*I–K*). Because the abnormalities observed in *Aph-1a*^{-/-} embryos resemble those of *Notch1*, *nicastrin*, or *PS* null embryos and together with observations that *Aph-1b* null mice (our unpublished data) and *Aph-1c* null mice (B. De Strooper, personal communication) are viable and show no apparent overt phenotype, our results establish that APH-1a is the principal APH-1 isoform required for Notch signaling during mammalian embryogenesis.

APH-1a is the major APH-1 isoform required for the assembly of the γ -secretase complexes and APP processing

To assess the role of APH-1a in γ -secretase complex formation and APP processing, we established primary and immortalized fibroblasts from E9.5 control, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} embryos. We initially confirmed that APH-1aL was absent in *Aph-1a*^{-/-} fibroblasts, whereas in *Aph-1a*^{+/-} cells, APH-1aL accumulated to ~50% of the level found in *Aph-1a*^{+/+} cells (Fig. 3*A*). To determine the contribution of APH-1a in γ -secretase complex formation, we examined levels of nicastrin, PS, and PEN-2 in E9.5 *Aph-1a*^{-/-} immortalized fibroblasts by protein blot analysis. We found that levels of mature nicastrin, PS1-CTF, PS2-CTF, and PEN-2 were significantly reduced in *Aph-1a*^{-/-} cells compared with controls (Fig. 3*A*). Moreover, formation of high-molecular-weight γ -secretase complexes was dramatically decreased in *Aph-1a*^{-/-} fibroblasts (Fig. 3*B*), as judged by immunoblotting of blue native gel using the nicastrin-specific or PS1-specific antibodies. Importantly, the formation of APH-1/nicastrin subcomplex was also significantly reduced in *Aph-1a* null cells (Fig. 3*B*). Together with *Aph-1* RNAi studies (Lee et al., 2002; Shirotani et al., 2004), our results strongly suggest that APH-1a is required to form a stable subcomplex with nicastrin to facilitate the assembly of PS and PEN-2, leading to the formation of mature γ -secretase complex.

To confirm that the reduction in level of high-molecular-weight γ -secretase complex observed in *Aph-1a*^{-/-} fibroblasts directly impacts on APP processing and leads to a decrease in secretion of A β peptides, we infected *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} fibroblasts with recombinant adenovirus expressing a humanized APP cDNA bearing the Swedish variant (APPSwe) (Cai et al., 2001). Protein blot analysis using CT15, an antibody specific for the C terminus of APP, revealed an accumulation of APP C-terminal fragments relative to full-length APP in *Aph-1a* null cells compared with control fibroblasts (Fig. 4*A*). Furthermore, quantitative sandwich ELISA analysis of conditioned media from *Aph-1a*^{-/-} cultures expressing APPSwe showed, respectively, 80.4 \pm 8.3 and 78.8 \pm 7.4% reduction in the levels of A β 40 and A β 42 peptides (Fig. 4*B*). Together, these results establish that APH-1a is the principal mammalian APH-1 isoform required for assembly of γ -secretase complex during embryonic development.

All APH-1 isoforms complement reduced levels of components of γ -secretase complexes and of secretion of A β in *Aph-1a*^{-/-} cells

Because there are three mouse *Aph-1* alleles that encode four distinct APH-1 isoforms, it is not clear whether APH-1 isoforms possess similar or distinct functions. To examine the functional differences among these variants, we initially performed complemen-

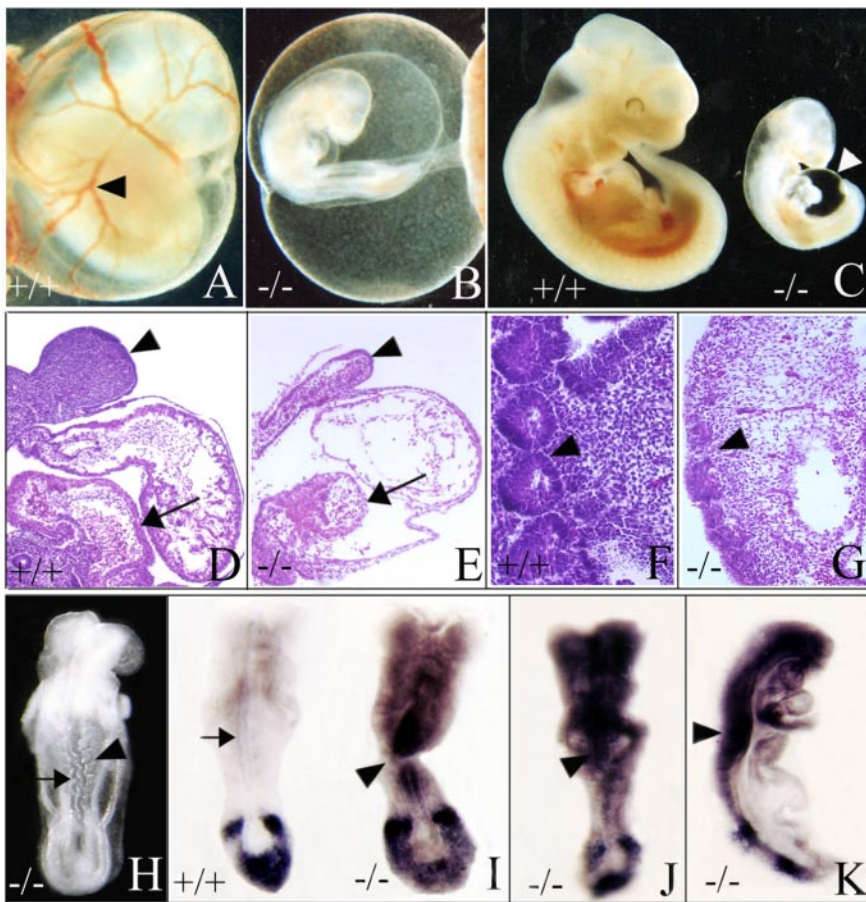


Figure 2. *Aph-1a*^{-/-} embryos exhibit a *Notch1*-like phenotype. *A, B*, Yolk sac vasculature is abnormal in *Aph-1a*^{-/-} embryos. Whereas normal yolk sac vasculature (arrowhead) is observed at E9.5 in *Aph-1a*^{+/+} embryos (*A*), *Aph-1a*^{-/-} embryos exhibit abnormal vascular morphogenesis of the yolk sac (*B*). *C*, The development of *Aph-1a*^{-/-} embryos is dramatically retarded by E10.5 compared with littermate controls. White arrowhead points to distention of pericardial sac in *Aph-1a*^{-/-} embryos. *D–G*, Sagittal sections of *Aph-1a*^{+/+} (*D, F*) and *Aph-1a*^{-/-} (*E, G*) embryos stained with H&E. Compared with littermate controls (*D*), *Aph-1a*^{-/-} embryos (*E*) exhibit patterning defects in the first branchial arch (arrowheads) and the heart chamber (arrows). In contrast to normal somite segmentation observed in *Aph-1a*^{+/+} embryos (*F*), defective patterning of somites (arrowheads) is seen in *Aph-1a*^{-/-} embryos (*G*). *H*, E8.5 *Aph-1a*^{-/-} embryos showed kinked neural tube (arrow), in addition to abnormal patterning of somites (arrowhead). *I–K*, Whole-mount *in situ* hybridization of *Dll1*. Whereas *Dll1* expression is not detected in neural tubes of *Aph-1a*^{+/+} embryos (arrow in *I*), increased expression of *Dll1* is observed in *Aph-1a*^{-/-} embryos (arrowheads in *J–K*).

tation experiments by expressing different APH-1 isoforms in *Aph-1a* null fibroblasts and determined the accumulation of NCT, PS1, and PEN-2. Compared with *Aph-1a*^{-/-} fibroblasts, the levels of mature NCT, PS1-CTF, and PEN-2 were all significantly elevated in *Aph-1a*^{-/-} cells expressing, respectively, APH-1aL, APH-1a5-V5, APH-1b-V5, or APH-1c (Fig. 5A). Moreover, the levels of secreted A β 40 from *Aph-1a*^{-/-} fibroblasts expressing any of the four APH-1 isoforms were also significantly increased as analyzed by quantitative sandwich ELISA (Fig. 5B). These results demonstrated that APH-1 isoforms are functional and facilitate formation of γ -secretase complex and support the view that APH-1 isoforms define a set of distinct functional γ -secretase complexes.

Discussion

An emerging view is that PSs form high-molecular-weight complexes with three transmembrane proteins (namely, nicastrin, APH-1, and PEN-2) that are critical for generation of functional γ -secretase complexes (De Strooper, 2003). However, the exact roles of these proteins, particularly for APH-1 in mammals in which three homologous genes exist, in regulation of γ -secretase complex assembly remain incompletely defined. Despite the

demonstration that APH-1 and PEN-2 play important roles in PS-mediated Notch signaling and APP processing (Francis et al., 2002; Goutte et al., 2002) and that PS, nicastrin, APH-1, and PEN-2 comprise the core γ -secretase complex (Edbauer et al., 2003; Farmery et al., 2003; Kim et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003), the exact mechanism whereby the four components are assembled into the final active complex is ill defined. Particularly intriguing is what physiological roles the three murine *Aph-1* genes that encode four APH-1 homologs have. To begin to address this issue, we took a genetic approach to ablate the murine *Aph-1* genes. Our demonstration that the phenotype of *Aph-1a* null embryos resembles that of *Notch1* null (Swiatek et al., 1994; Conlon et al., 1995; Huppert et al., 2000), *PS* null (Donoviel et al., 1999; Hererman et al., 1999), or *nicastrin* null (Li et al., 2003) embryos not only confirms that APH-1a is required for γ -secretase mediated Notch signaling during mammalian development but establishes that APH-1a is the principal mammalian APH-1 homolog in presenilin-dependent γ -secretase complexes required for embryonic development. The fact that APH-1b or APH-1c was not able to compensate for the lack of APH-1a in *Aph-1a*^{-/-} mice indicates that the expression levels of *Aph-1b* and *Aph-1c* genes might be very low or that they are differentially expressed in different cell types during early embryogenesis. Consistent with the idea that APH-1b/c is expressed to low levels during embryogenesis is the finding that the *Aph-1a* mRNA is expressed at much higher levels than those of *Aph-1b* and *Aph-1c* (see the University of California, Santa Cruz mouse gene sorter available at www.genome.ucsc.edu/cgi-bin/hgNear and microarray data).

Because the phenotype of *Aph-1a*^{-/-} mice is not identical to *Notch1* null embryos, the low level of APH-1b and APH-1c would contribute to γ -secretase activity during embryonic development, and it may be anticipated that *Aph-1a*^{-/-}; *Aph-1b*^{-/-} or *Aph-1a*^{-/-}; *Aph-1c*^{-/-} embryos might phenocopy onto the *Notch1* null embryos. Indeed, analysis of *Aph-1a*^{-/-}; *Aph-1b*^{-/-} embryos revealed phenotypes that are nearly identical to *Notch1*-deficient embryos (T. Li, G. Ma, H. Wen, F. Davenport, D. L. Price, and P. C. Wong, unpublished observations). Together with the observation that *Aph-1a* mRNA are reduced during postnatal development (see the University of California, Santa Cruz genome website available at www.genome.ucsc.edu), our results raise the interesting possibility that mammalian *Aph-1* genes are developmentally regulated. Recent studies indicated that mammalian APH-1 isoforms are differentially expressed in different organs–cell types in the adult animal (Hebert et al., 2004). The identification of a specific APH-1 isoform selectively expressed in the CNS during aging would have important therapeutic implica-

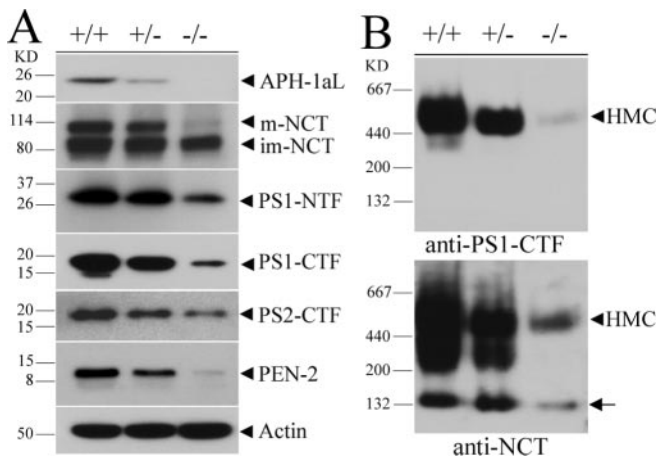


Figure 3. Levels of nicastrin, PS1, PEN-2, and γ -secretase complex formation in *Aph-1a*^{-/-} cells. *A*, Protein extracts from immortalized *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} fibroblasts were subjected to protein blot analysis using antisera specific to APH-1aL, NCT, PS1-NTF, PS1-CTF, PS2-CTF, PEN-2, and actin. *B*, Membrane fractions of *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} cells were prepared and subjected to blue native gel analysis using anti-NCT and anti-PS1 antibody. Both the levels of high-molecular-weight complex and APH-1/nicastrin subcomplex were reduced in *Aph-1a*^{-/-} cells. HMC, High-molecular-weight γ -secretase complexes; arrow indicates APH-1/nicastrin subcomplexes.

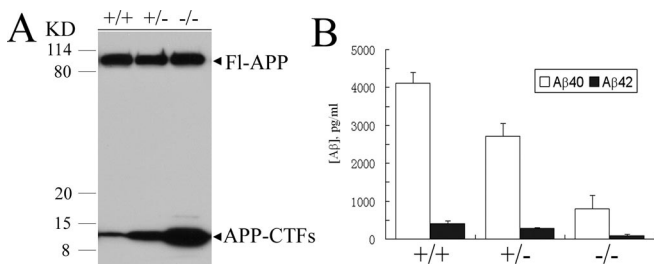


Figure 4. APP processing and secretion of A β in *Aph-1a*^{-/-} fibroblasts. *A*, *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} fibroblasts were infected with adenovirus expressing the human APP Swedish mutation for 36 hr, and extracts were subjected to protein blot analysis using CT15, an anti-APP C-terminal antibody. FI-APP, Full-length APP; APP-CTFs, APP C-terminal fragments. The ratio of the APP-CTFs to full-length APP is significantly increased in *Aph-1a*^{-/-} cells. *B*, Conditioned media from *Aph-1a*^{+/+}, *Aph-1a*^{+/-}, and *Aph-1a*^{-/-} fibroblasts were collected and analyzed by sandwich ELISA. Levels of secreted A β 40 and A β 42 were significantly reduced in *Aph-1a*^{-/-} cells.

tions for efforts to selectively alter γ -secretase activity to ameliorate β -amyloidosis in AD.

Although previous studies suggested that APH-1 and nicastrin form a precomplex that might facilitate assembly of the γ -secretase complex (Gu et al., 2003; LaVoie et al., 2003; Hu and Fortini, 2003; Takasugi et al., 2003), the molecular mechanism of precomplex assembly and whether APH-1 homologs are required to stabilize nicastrin to form the initial pre- γ -secretase complex remain elusive. Our findings that the deletion of *Aph-1a* significantly reduces the levels of mature nicastrin as well as APH-1/nicastrin precomplex and high-molecular-weight complex coupled with the observation that APH-1 and nicastrin physically interact (Gu et al., 2003; Hu and Fortini, 2003; LaVoie et al., 2003; Morais et al., 2003) strongly support the view that APH-1 is necessary to stabilize nicastrin to form a stable precomplex. Together with the observation that the level of APH-1 is significantly reduced in *nicastrin*-deficient fibroblasts (Vetrivel et al., 2004), our data are consistent with a model that APH-1 and nicastrin are

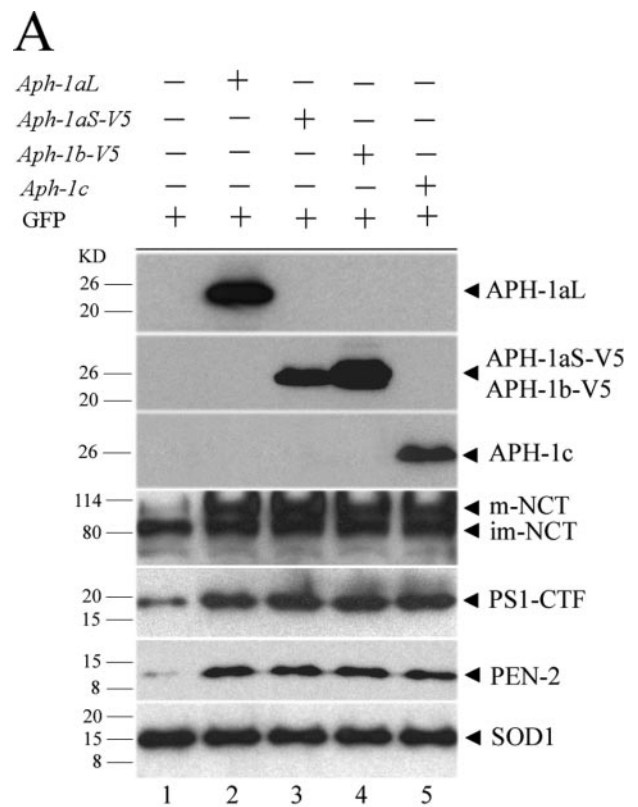


Figure 5. Reduced levels of the components of γ -secretase complex and enzymatic activity occurring in *Aph-1a*^{-/-} cells can be complemented by expression of any APH-1 isoforms. *A*, Immortalized *Aph-1a*^{-/-} cells transiently transfected with expression plasmids encoding APH-1aL, APH-1aS-V5, APH-1b-V5, or APH-1c were infected with APP virus expressing human APP Swedish mutation 24 hr after transfection. Cell extracts were prepared 48 hr after transfection and subjected to protein blot analysis using anti-NCT, anti-PS1, and anti-PEN-2 antibodies. Cotransfection of expression vector encoding GFP (green fluorescent protein) was performed to estimate the efficiency of transfection (30–40%). The expression of APH-1 isoforms was analyzed using anti-APH-1aL antibody for APH-1aL, anti-V5 antibody for APH-1aS and APH-1b, and anti-APH-1b/c for APH-1c. The blots were reprobbed using antisera specific to SOD1. *B*, Conditioned media were collected 48 hr after transfection (24 hr after infection) and analyzed by quantitative sandwich ELISA. The levels of secreted A β 40 were significantly elevated in cultures of cells overexpressing APH-1aL, APH-1aS-V5, APH-1b-V5, or APH-1c.

required to regulate the stability of each other to form a stable precomplex that serves as a platform for assembling PS and PEN-2 to form a core γ -secretase complex.

Although recent studies showing that increased levels of active

γ -secretase complex can be achieved by overexpression of APH-1, PEN-2, and nicastrin with PS (Kim et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003) and that γ -secretase activity can be reconstituted in yeast (Edbauer et al., 2003) support the view that these four components constitute the minimal γ -secretase complex, it is not clear whether APH-1 isoforms serve to define a set of distinct γ -secretase complexes. Recent biochemical studies suggest that there may exist several distinct presenilin complexes (Gu et al., 2004) with presenilin dimer forming the core of the γ -secretase enzyme (Schroeter et al., 2003) and that six distinct γ -secretase complexes with different APH-1 isoforms can exist in cultured cells (Shirotani et al., 2004). Our demonstration that all murine APH-1 isoforms can complement the *Aph-1a* null phenotype in fibroblasts supports the idea that the four murine APH-1 isoforms (APH-1aL, APH-1aS, APH-1b, and APH-1c) coupled with PS1 and PS2 define a set of eight distinct functional γ -secretase complexes. Consistent with this view is our finding that *Aph-1b*-deficient fibroblasts showed reduced levels of nicastrin, PS, and PEN-2, and, more importantly, the level of γ -secretase activity is directly proportional to the *Aph-1* gene dosage (Li, Ma, Wen, Davenport, Price, and Wong, unpublished observations). Future studies directed at defining the role of APH-1 dependent γ -secretase complexes, particularly specific APH-1 isoform that may determine cleavage site selectivity on APP-CTF, would have important implications for development of specific anti-A β therapy for AD.

In summary, the present investigation has established that APH-1a is the major APH-1 isoform required for γ -secretase activity during mouse embryonic development and that APH-1a is critical to stabilize nicastrin to form a pre- γ -secretase complex that facilitates the assembly of PS and PEN-2. It remains to be demonstrated whether a specific APH-1 isoform might be a valuable therapeutic target for AD, a possibility that can be evaluated in mouse models of A β amyloidosis.

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